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13. ABSTRACT ***(Maximum 200 Words)***

We studied the mechanisms of regulation of the FGF binding protein (FGF-BP) in breast cancer cells and evaluated its function in initial studies as a transgene in mice.

We demonstrate that the FGF-BP gene is regulated at the transcriptional level in MDA-MB 468 cells. The promoter elements contributing are delineated.

Initial studies with transgenic animals show that the FGF-BP gene expressed under a constitutively active promoter (CMV, K14, MMTV) causes embryonic lethality around mid-gestation time.

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Table of Contents

INTRODUCTION

The potential role of the secreted binding protein for FGF (BP) as a carrier for immobilized FGFs:

One mode of delivering active FGF from the storage site to its receptor could be binding to a secreted carrier protein. The secreted FGF binding protein (BP) can serve as such an an extracellular chaperone molecule for FGFs[1].

The major focus of this cycle was to explore the genetic basis for the activation of FGF-BP transcription during breast cancer progression and we used a cell model for that. Also, we initiated transgenic animal studies. We pursued the following aims:

Technial objectives:

Goal (3): To determine if the differential expression of FGF-BP in breast cancer cell lines is controlled at the transcriptional levels by examining the regulation of the FGF-BP gene promoter in cell lines.

Goal (4): To examine the direct impact of FGF-BP overexpression in transgenic animals.

BODY

TASK 3:

To determine if the differential expression of FGF-BP in breast cancer cell lines is controlled at the transcriptional levels by examining the regulation of the FGF-BP gene promoter.

Work accomplished during the second award cycle (Months 12 – 24):

A series of FGF-BP promoter/ luciferase reporter constructs were tested for their basal activity and their EGF induction in MDA-MB 468 cells. Figure 1 shows the full length promoter as well as different deletion constructs to dissect out, which portions of the promoter are the most significant for basal and EGF induction.

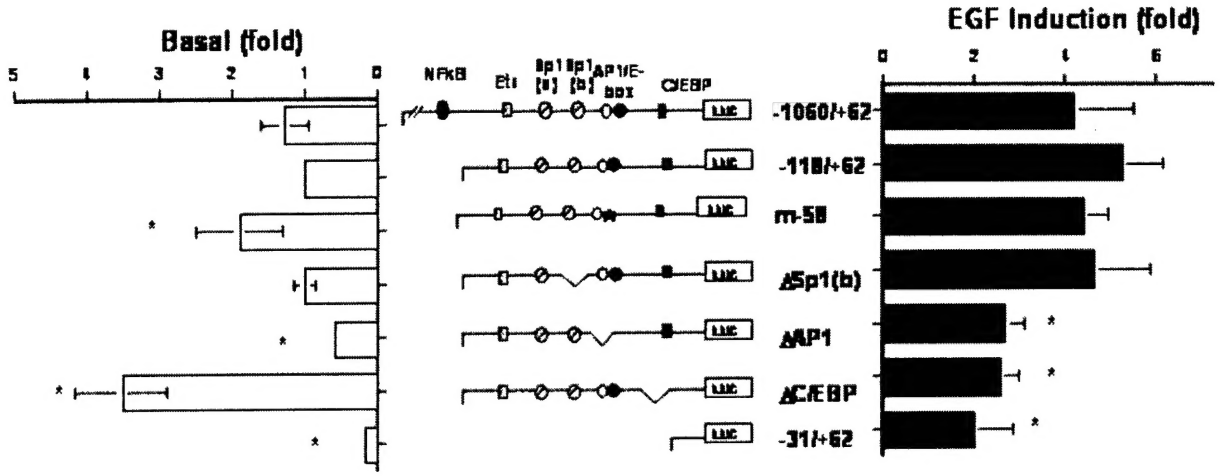

Figure 1. **FGF-BP promoter elements involved in EGF induction of FGF-BP in MDA-MB-468 cells.**
The *histogram* on the *left* shows the impact of each promoter deletion on the basal (uninduced) luciferase activity of each construct. The basal activity of the -118/+62 construct was set to 1. The *right* histogram shows the transcriptional activity in the presence of EGF and is expressed as -fold induction of EGF-treated over untreated for each construct. MDA-MB-468 cells were transiently transfected by electroporation with the indicated FGF-BP promoter luciferase constructs, and a CMV driven *Renilla* luciferase reporter vector for transfection efficiency, and were untreated or treated with 10 ng/ml of EGF for 18 h. Promoter constructs are described under "Methods" and in Ref. [2]. Values represent the mean and S.E. from at least three separate experiments, each done in triplicate wells. Statistically significant differences relative to the –118/+62 promoter construct are indicated (*, $p<0.05$, t test).

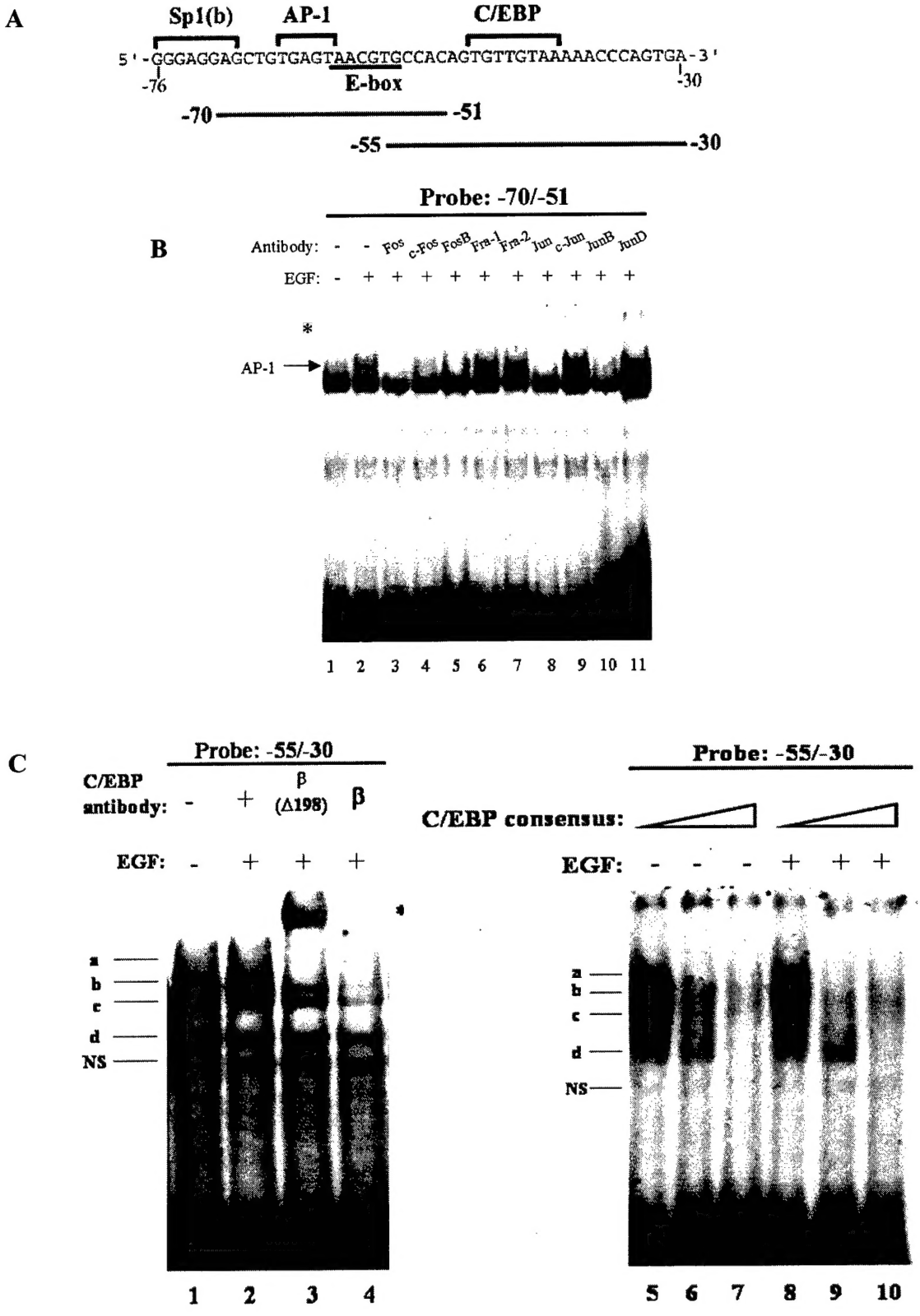

Figure 2. **Transcription factor binding to FGF-BP promoter ... (FULL TEXT OF THE LEGEND ON THE NEXT PAGE).**

Figure 2 (!graph on previous page !). **Transcription factor binding to FGF-BP promoter elements in MDA-MB-468 cells.** *A,* Double-stranded oligonucleotide sequences of promoter elements used for gel shift analysis. *B,* Supershift analysis of transcription factor binding to the AP-1, and *C,* C/EBP sites of the FGF-BP promoter. Labeled FGF-BP promoter sequences as indicated were incubated with nuclear extracts from untreated or EGF-treated MDA-MB-468 cells. Binding reactions were performed in the presence of "supershifting" antibodies as indicated. An arrow or bar to the left of each panel indicates specific binding of AP-1 and C/EBP. Asterisks indicate supershifted complexes.

TASK 3; Methods:

Cell Culture and Reagents

The MDA-MB-468 human breast cancer cell line, and the ME-180 human cervical squamous cell carcinoma cell line were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in improved minimum essential medium (IMEM) with 10% fetal bovine serum (Invitrogen Inc.; Carlsbad, CA). Human recombinant EGF was purchased from Collaborative Biochemical Products (Bedford, MA). Tyrphostin AG1517 (PD153035), Ro 31-8220 (bisindoylmaleimide IX) and PP1 were purchased from Alexis Corp. SB202190 was purchased from Calbiochem (San Diego, CA). U0126 was purchased from Promega. Calphostin C was purchased Sigma-RBI (Natick, MA). Wortmannin was purchased from Biomol (Plymouth Meeting, PA). All compounds were dissolved in Me_2SO.*Plasmids*- Human FGF-BP promoter fragments were cloned into the pXP1 promoterless luciferase reporter vector and have been described previously [2]. The MEK2 (K101A) dominant negative construct was provided by Dr. J. Holt (Vanderbilt University). The expression plasmids containing wild-type p38 (pCDNA3-Flag-p38), and constitutively active MKK6 (pCDNA3-Flag-MKK6(Glu)) were provided by Dr. R. Davis (University of Massachusetts). The expression vectors for human CEBPb-LAP and C/EBPb-LIP (CMV-LAP and CMV-LIP, respectively) were gifts from Dr. U. Schibler (University of Geneva) courtesy of Dr. J. Schwartz (University of Michigan). Wildtype C/EBPb mRNA contains three inframe AUG translation start sites, from which LAP and LIP are translated from the second and third sites, respectively [3]. The second in frame AUG is flanked by an imperfect Kozak's sequence, GACCATGG [4], compared to the Kozak's consensus sequence of CCA/GCCAUGG [5,6], whereas the third inframe AUG is flanked by a perfect Kozak's sequence [3], resulting in translation of both LAP and LIP. The CMV-LAP construct contains only the second and third translation start sites, but both are flanked by perfectly matched Kozak's sequences resulting in the more efficient translation of LAP alone [3]. The effects of dominant negatives or activated constructs were compared to their empty vector control or with the empty vector pCDNA3 (Invitrogen).

Transient Transfections and Reporter Gene Assays- Twenty-four hours before transfection MDA-MB-468 cells were plated at a density of $3x10^6$ cells in 10-cm dishes. pRL-CMV *Renilla* luciferase reporter vector (Promega; Madison, WI) was included as a control for transfection efficiency. MDA-MB-468 cells were transfected by electroporation as described by Raja *et al.* [7]. Briefly, cells were trypsinized and washed twice by centrifugation in IMEM containing 10% FBS. The cells from each plate were then resuspended in 400 μl IMEM containing 20% FBS. A total of 30 μg plasmid DNA (29 μg of FGF-BP promoter construct, 3.0 ng of pRL-CMV) was added to the cell suspension 5 minutes before electroporation. For co-transfection, 24 μg of -118/+62Luc FGF-BP promoter construct, 5 μg or indicated amounts of expression vector, and 3.0 ng of pRL-CMV were added to cells. Electroporation of the entire cell sample was carried out in a cuvette with an electrode gap of 0.4 cm at 350 V and 500 mF, using a BioRad GenePulser II (Bio-Rad; Hercules, CA). The electroporated cells were then distributed equally to a 6-well plate, each well having been pre-filled with 3 ml of IMEM with 10%

FBS. Cells were allowed to recover and attach for 16 hours before treatment. Transfected cells were washed twice with serum-free IMEM, treated with or without EGF (10 ng/ml) in serum-free IMEM for 16 hours, and then lysed in 150 μl of passive lysis buffer (Promega). 20 μl of extract was assayed for both firefly and *Renilla* luciferase activity using the Dual-Luciferase™ reporter assay system (Promega). To correct for transfection efficiency, and a small background induction (1.5-2.0 fold) of the pRL-CMV plasmid by EGF [8], *Renilla* luciferase values were corrected for protein content, and these numbers were then used to normalize firefly luciferase values. Protein content of cell extracts was determined by Bradford assay (Bio-Rad).

Gel Shift Assays- MDA-MB-468 cells were grown to 80% confluence on 150-mm dishes, serum starved for 16 hours, and treated with or without 10 ng/ml EGF for 1 hour. As a control, ME-180 SCC cells were treated with 5 ng/ml EGF for 1 hour. For gel shift assays using transiently transfected cells, MDA-MB-468 cells were plated at a density of $6x10^6$ cells in 150-mm dishes, and transfected with 10 mg of expression vector or empty vector by electroporation as described above. Cells were then either untreated or treated with 10ng/ml EGF for 1 hour. To study the effects of inhibition of p38 MAPK, cells were pretreated for 1 hour with 10 μM SB202190, then treated with or without 10 ng/ml EGF for 1 hour. Nuclear extracts were prepared as described previously [2]. Binding reactions with the -70/-51 and -55/-30 probe was carried out as described previously [2] with 6 μg of MDA-MB-468 nuclear extracts, binding buffer (20 mM Tris, pH 7.5, 60 mM KCl, 5% glycerol, 0.5 mM dithiothreitol, 2.0 mM EDTA), and 500 ng of poly(dI-dC).

Supershift antibodies (2 μg) were added to the binding reaction for 10 minutes on ice before adding 20 fmol of labeled probe. Reactions were carried out for 20 minutes at room temperature and analyzed by 6% polyacrylamide gel electrophoresis. Fos-specific antibodies c-Fos (K-25), c-Fos (4), Fos B (102), Fra-1 (R-20), and Fra-2 (Q-20); Jun-specific antibodies c-Jun/AP-1 (D), c-Jun/AP-1 (N), JunB (N-17), and JunD (329); and C/EBP specific antibodies C/EBPb (C-19), and C/EBPb (D198) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistics – The GraphPad/Prism software package was used for graphics and data evaluation. ANOVA was applied for continuous variables and chi-square (Fisher's exact test) for discontinuous variables. p. values < 0.05 were considered significant.

TASK 3; INTERPRETATION OF THE DATA

Mutational analysis revealed that the AP-1 and C/EBP sites on the FGF-BP gene promoter were required for the EGF effect. Surprisingly, deletion of the C/EBP site resulted in a significant increase in promoter basal activity. This indicates a basal repressive control mechanism (Figure 1).

This transcriptional activity assay was corroborated by gel shift analysis showing binding of transcription factors to the C/EBP site (Figure 2).

In composite these data suggest that the C/EBP site is a central regulatory element for the regulation of FGF-BP promoter activity in MDA-MB-468 cells.

TASK 4:
To examine the direct impact of FGF-BP overexpression in the mammary epithelium using transgenic animals.

We hypothesize that expression of FGF-BP as a transgene in a time- and tissue-dependent manner will shed light on its function in the intact organism, in particular the mammary gland. It was planned to generate transgenic animals and then study the impact on the mammary gland phenotype.

Work accomplished during the second award cycle (Months 12 – 24):

We planned to generate transgenic mouse lines and used three distinct promoters driving FGF-BP.

CMV-FGF-BP as a transgene:
This construct was used earlier in studies in cell lines to expresss the human FGF-BP and study its function in animal tumor growth [1]. We found in initial studies with this construct in transgenic animals that human FGF-BP (hFGF-BP) transgene expression in mice using the CMV promoter/enhancer leads to embryonic lethality. A number of separate preparations of the vector were used but still no viable, transgenic offspring were obtained in a large series of embryo transfers (>20).
The time of loss of embryos was around e12 to e15 and the cause of the loss of embryos was due to bleeding into the embryonic sac. We concluded that perhaps the widespread expression would cause toxicity to the embryo and thus generated two different vectors with a more limited expression pattern, K14- and MMTV-hFGF-BP

MMTV-hFGF-BP as a transgene:
In a separate experimental series we used the MMTV-LTR (abbreviated with"MMTV") to drive hFGF-BP expression as a transgene since its timing and tissue-specificity of expression is distinct from the K14-hFGF-BP. The MMTV-LTR promoter/enhancer construct was provided by our transgenic facility who obtained it from Dr. Phil Leder's laboratory. Studies by other laboratories have shown that the activates expression of a transgene in mostly epithelial tissues and as early as the four-cell-stage of embryogenesis. In two separate experiments with the MMTV-hFGF-BP transgene construct with a total of seven foster mothers and more than 20 embryos per foster mother, only 5 viable offspring were obtained. None of these were transgenic for hFGF-BP. From our preliminary assessment of these experiments the loss of the pregnancies also occurred before day e15. Whether implantation occurred was not checked in these two experiments.

K14-FGF-BP as a transgene:
Based on the above data with CMV-FGF-BP we decided to utilize a more restricted promoter for expression of the transgene, i.e. K14. In 5 independent embryo transfer experiments with 2 to 3 foster mothers in each of the experiments and more than 20 embryos per foster mother, we only obtained a total of 10 live offspring. None of these were transgenic for hFGF-BP as assessed by genomic Southern blotting and PCR.

However, the K14-hFGF-BP injected embryos did implant as evidenced by histologic sectioning of uteri from foster mothers that had failed to generate viable offsprings. Monitoring of the pregnant mice showed that the pregnancies were lost prior to day 15.

Expression profile of the endogenous murine FGF-BP:
To compare the effect to the endogenous FGF-BP time course we ran a series of staining in mouse embryos. We found that endogenous mFGF-BP mRNA is barely above background on embryonic day e9, can be detected by day e12 and reaches its peak expression prenatally (day e16/17). The relative expression levels at these three time points are 1 / 4 / 20 for days e9 / e12 / e17 respectively (see Ref. [9]). Most of the expression at day 12 was found in the basal layer of the skin and in the gut as assessed by in situ hybridization.

Conclusion: Obviously expression of hFGF-BP under the control of CMV, K14 or MMTV is embryonically lethal. Endogenous murine FGF-BP expression is initiated after day e8 at low levels and peaks much later, i.e. prenatally. Gut, lung and skin are the major tissues of expression of the endogenous gene.

TASK 4; INTERPRETATION OF THE DATA
We found that expression of FGF-BP as a transgene in mice results in early embryonic lethality in due to hemorrhage into the gestational sac. K14, MMTV or CMV-dependent transgene expression resulted in qualitatively the same phenotype that was only distinguished by a slightly different onset between days.

Due to this unexpected lethality of FGF-BP expression, we decided to initiate generation of tetracycline-regulatable FGF-BP expression in transgenic animals.

KEY RESEARCH ACCOMPLISHMENTS

1. Analysis of the promoter elements driving EGF regulation of the FGF-BP gene in the breast cancer cell model.
2. Analysis of the transcription factor binding to the FGF-BP promoter.
3. Finding that FGF-BP causes embryonic lethality during the second half of gestation of transgenic mice with K14, MMTV and CMV promoters used to drive FGF-BP transgene expression.

REPORTABLE OUTCOMES

Manuscripts (in preparation):

Kagan et al. On the transcriptional regulation of FGF-BP in Breast Cancer.

CONCLUSIONS

- The FGF-BP gene at the transcriptional level utilizes the transcription factor binding site and proteins from the C/EBPbeta family.

- The FGF-BP transgene is lethal when expressed during embryogenesis in mice.

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APPENDICES

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